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Minireview

RNA interference as a new strategy against viral hepatitis

Senthil K. Radhakrishnan, Thomas J. Layden, and Andrei L. Gartel*

Department of Medicine, University of Illinois at Chicago, Chicago, IL 60612, USA

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Abstract

Hepatitis viruses are the leading cause of liver cirrhosis and hepatocellular carcinoma worldwide. Since currently available treatment options against these viruses are limited, there is a need for development of alternative therapies. In this minireview, we concentrate on three hepatitis viruses—hepatitis C virus, hepatitis B virus, and hepatitis delta virus and discuss how RNA interference (RNAi) has been utilized against them. RNAi is a process by which small double-stranded RNA can effectively target a homologous RNA sequence for degradation by cellular ribonucleases. Though RNAi was exploited in the beginning for down-regulating cellular genes, it has recently been demonstrated that this process is equally effective against many types of human and animal viruses including the hepatitis viruses. Both synthetic small-interfering RNAs (siRNAs) and plasmid-based siRNA expression systems have been useful in suppressing the hepatitis viruses. Though this new approach looks promising, problems of nonspecific effects and delivery may need to be addressed before the full therapeutic potential of RNAi against viral infections in patients is realized.

Keywords: RNA interference; Viral hepatitis; dsRNA

Introduction

RNA interference (RNAi) is a process of sequence-specific post-transcriptional gene silencing initiated by double-stranded RNA (dsRNA). This phenomenon was first observed in the nematode *Caenorhabditis elegans* (Fire et al., 1998) and is conserved in mammalian cells. RNAi-dependent silencing in *C. elegans* can be initiated through dsRNA injection, soaking of worms in dsRNA, or feeding the worms with dsRNA-producing bacteria. After delivery of long dsRNA to *C. elegans*, it is subsequently processed into 21–25 bp functional small interfering RNA (siRNA) by an enzyme called Dicer that belongs to the RNase III family (Bernstein et al., 2001). siRNAs are incorporated into an enzyme complex RISC (RNA-induced silencing complex), which upon activation unwinds the siRNA. This unwound siRNA is used by RISC for selecting the target RNA by Watson–Crick base-pairing, which is later degraded in the region of homology directed by the original siRNA (Elbashir et al., 2001).

The process of RNAi has been extended recently to mammalian cells with a few modifications. Unlike in *C. elegans*, long dsRNA cannot be used in mammalian systems because they evoke a nonspecific interferon (IFN) response that activates protein kinase PKR (Balachandran et al., 2000). But this problem has been circumvented recently by using short RNA duplexes of length approximately 21 bases with 2- or 3-nucleotide (nt) 3′-end overhangs. Elbashir et al. (2001) successfully used this method against endogenous lamin A/C genes and tumor suppressor p53. Since then this method has been used to silence a large number of endogenous mammalian genes (for a review, see Hannon, 2002; McManus et al., 2002; Paddison and Hannon, 2002; Zamore, 2001).

Apart from silencing of cellular genes, RNAi is a very attractive option for suppressing viral RNA. In fact in plants, RNAi is a natural defense mechanism against RNA of invading viruses. The presence of a double-stranded RNA intermediate during the replication of the virus is thought to

* Corresponding author. Department of Medicine, University of Illinois at Chicago, 840 S Wood Street, Room 1041, Chicago, IL 60612. Fax: +1-312-355-2643.

E-mail address: agartel@uic.edu (A.L. Gartel).

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invoke the RNAi machinery. Recently, RNAi has been successfully used against several viruses in mammalian cells. Bitko and Barik (2001) used siRNA to silence the mRNAs produced by the respiratory syncytial virus (RSV), a negative strand virus that causes a form of respiratory disease. However, authors of this work could not inhibit the full-length viral genomic strand that might be due to the fact that the RNA was associated with structural proteins. Recently, HIV has also been targeted in several studies. In several in vitro models, siRNAs were directed either against HIV RNA (Capodici et al., 2002; Coburn and Cullen, 2002; Hu et al., 2002; Jacque et al., 2002; Lee et al., 2002; Novina et al., 2002; Park et al., 2002; Surabhi and Gaynor, 2002; Yamamoto et al., 2002) or were targeted against RNA encoding for the primary HIV-1 co-receptor, CXCR4/CCR5 (Cordelier et al., 2003; Martinez et al., 2002; Qin et al., 2003). RNAi has also been used against several other viruses including Dengue virus (Adelman et al., 2001, 2002; Caplen et al., 2002), flock house virus (FHV) (Li et al., 2002), rhesus rotavirus (RRV) (Dector et al., 2002), Semliki forest virus (SFV) (Caplen et al., 2002), influenza virus (Ge et al., 2003), and poliovirus (Gitlin et al., 2002).

It is also interesting to note that a few viruses have evolved counter-defenses against RNAi. It has been demonstrated that hepatitis delta virus (HDV) RNA is resistant to Dicer action (Chang et al., 2003). In *Drosophila* cells, it has been shown that FHV suppresses RNAi (Li et al., 2002). More recently, it was found that E3L and NS1 protein products encoded by the mammalian vaccinia and influenza viruses can suppress RNA silencing (Li et al., 2004). Also in plants, it is known that proteins such as 2b of Cucumovirus, AC2 of Geminivirus, HcPro of Potyvirus, P1 of Sobemovirus, and p19 of Tombus virus suppress gene silencing (Brigneti et al., 1998; Kasschau and Carrington, 1998; Vaucheret et al., 2001; Voinnet et al., 1999). The mechanisms of these processes are being unraveled gradually. For example, the precise structural basis underlying the sequence-independent recognition and sequestration of 19–21 nt siRNAs by p19 of Tombus virus has been demonstrated recently by X-ray crystallography (Ye et al., 2003).

RNAi against hepatitis C virus

Hepatitis C virus (HCV) is a member of *Flaviviridae* viruses that replicate mainly in the liver of infected patients. HCV is classified into six major genotypes differing more than 30% from each other in their nucleotide sequence. The prototype strain of HCV genotype 1a is found predominantly in the US and Northern Europe, and genotype 1b initially found in Japan now has worldwide distribution. HCV possesses a positive-strand RNA of about 9.6 kb consisting of the 5′ untranslated region (5′-UTR), the open reading frame (ORF), and the 3′-UTR (Fig. 1). Because the HCV genome is a single-stranded RNA that serves also as a messenger RNA, it is an appealing target for developing RNAi-based therapies.

The 5′-UTR is a 341-nucleotide (nt) sequence that is highly conserved even between the most distantly related HCV subtypes. The ORF produces a polyprotein that can subsequently be processed into at least 10 different proteins including a capsid (core) protein, two envelope proteins (E1 and E2), and nonstructural proteins (NS2, NS3, NS4, NS5A, and NS5B) (Fig. 1A). The NS5B protein is an RNA-dependent RNA polymerase (RdRP) and is the key component in HCV replication. It has been shown that NS5B associates with NS3 and NS4A to produce a negative-strand copy of the RNA genome, which in turn can give rise to several positive-strand RNA copies.

There are no vaccines available against HCV. Currently, the favored therapy for HCV infection is the use of PEG-interferon-α in combination with ribavirin. Over the last decade this therapy led to sustained virologic response of 40–80% of patients dependent on HCV genotype. Very recently, a small molecule (BILN 2061) inhibitor of NS3 protease has been shown to be more efficient than either interferon or ribavirin in restricted clinical trials of human patients (Lamarre et al., 2003). But it is too early to adopt this in standard therapy against HCV. Much of this struggle against HCV is due to its genetically heterogeneous nature coupled with the existence of quasispecies. Quasispecies are distinct but closely related variants of the virus that circulate

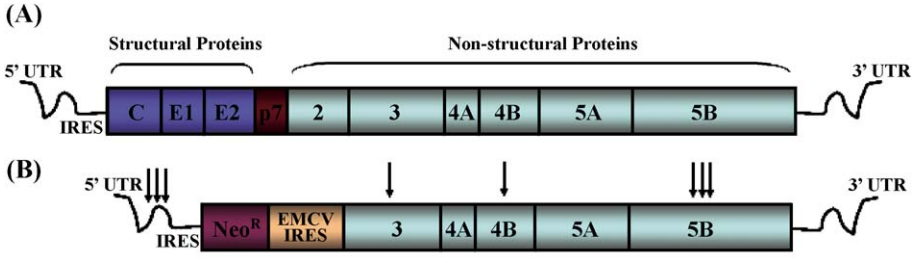

Fig. 1. Targets that have been used against HCV. (A) Organization of protein coding regions in the HCV RNA genome is shown. While the structural proteins consist of C, E1, and E2, the nonstructural proteins are NS2, NS3, NS4A, NS5A, and NS5B. At present, it is not known if p7 belongs to structural or nonstructural category. (B) Schematic representation of the subgenomic replicon that has been used in some studies described in the text is shown. Regions starting from C until NS2 have been removed and instead replaced with a Neomycin resistance gene and the EMCV IRES. The target regions that have been used by various groups are indicated by downward arrow.

in infected individuals. Viral heterogeneity results from high error rate of NS5B gene-coded RNA-dependent RNA polymerase, which gives significant advantage to HCV survival. The 5′-UTR region seems to be subjected to much stricter conservation than other regions of the HCV genome. For example, the sequences between 30 and 170 nt appear to be very much conserved between different quasispecies of HCV 1b genotype (Soler et al., 2002). Because RNA interference (RNAi) technology is very sensitive to single mismatches between siRNA and target sequences (Shi, 2003), the 5′-UTR appears to be the best target for silencing HCV.

Lack of in vitro cell culture models had been a major handicap for HCV research. But recently, subgenomic replicon systems for HCV have been developed (Fig. 1B) (Blight et al., 2000; Lohmann et al., 1999). These replicons have adaptive mutations that aid them to replicate efficiently in cell culture. They also lack the structural genes and so cannot produce active viral particles. Using HCV subgenomic replicon system, it has been shown recently that synthetic dsRNA can inhibit HCV RNA replication in cell culture. The specific targets on the HCV genome used in these studies are as shown in Table 1.

Seo et al. (2003) used a version of the HCV RNA subgenomic replicon that has a Neomycin resistance gene for selection and a luciferase gene for monitoring the levels of replicon expression. A reduction (85–90%) in the levels of luciferase was observed if cells were transfected with siRNAs specific for either the 5′-UTR or the luciferase, whereas nonspecific control siRNAs or siRNAs with three nucleotide mismatch to the luciferase target failed to show any reduction. To confirm that siRNAs do not produce cellular toxicity, they also measured cellular ATP levels and found them to be unchanged between transfected and mock-transfected cells.

Kapadia et al. (2003), using a subgenomic replicon system derived from HCV genotype 1b and siRNAs against NS3 and NS5B, showed 5.7- and 8.3-fold inhibition, respectively, as measured by real-time PCR 2 days after transfection. The levels of NS3 and NS5B proteins were found to be unchanged after 2 days as measured by Western Blot analysis, but started decreasing after day 4, suggesting that these proteins have a relatively long half-life. They also compared the extent to which HCV RNA replication was inhibited by RNAi and IFN treatment and found that siRNAs inhibited approximately 3-fold better than IFN and that the antiviral effect of siRNA is independent of IFN.

Randall et al. (2003), using a similar RNA replicon system, demonstrated about 5-fold decrease in 12 h and an 80-fold decrease in 96 h in HCV RNA levels as measured by real-time PCR when siRNAs against 5′-UTR were used. This level of total HCV RNA was still maintained after 8 days. The vast majority of the cells were cured of HCV RNA and protein beyond detectable levels by immunofluorescence with antibodies against NS5A. The number of G418-resistant colonies in siRNA-treated cells was dramatically decreased, supporting the conclusion that siRNAs mediate the clearance of replicating HCV RNA in this system. This work also showed high specificity of RNAi silencing of HCV replication because siRNAs that differed from the target sequence by only 3 nt failed to mediate suppression.

Wilson et al. (2003) selected both their siRNA targets against NS5B and showed about 90% reduction in HCV RNA levels 72 h post-transfection using Northern blot analysis. Also at this point, the nonstructural proteins NS3 and NS5B were below detectable levels as measured by immunoblotting. Next they produced siRNAs in a plasmid-based expression system, which expressed the sense and antisense strand of siRNA separately. Cells expressing siRNA were then selected and were challenged with HCV subgenomic RNA by electroporation. Three weeks later they found 70% less G418-resistant colonies in siRNA-expressing cells relative to control, suggesting that long-term suppression by RNAi may be achieved by this method.

Yokota et al. (2003) chose five targets against 5′-UTR and found that the most efficient siRNA, siRNA-331, suppressed HCV replication by 81% at a concentration of 2.5 nM and the suppression rate increased to 94% at 125 nM. On the basis of these results, they constructed DNA-based vectors for expressing siRNA-331. They either used a tandem type vector, where sense and antisense sequences were placed separately under the U6 promoter, or a stem-loop type vector, where the 3′ end of the sense sequence and the 5′ end of the antisense strand are connected by a 9-nt loop sequence and again placed under the control of U6

Table 1
Different targets that have been used to silence HCV

Authors	Target sequence	Target location
Seo et al. (2003)	5′ GUACUGCCUGAUAGGGUGC 3′	5′-UTR
Kapadia et al. (2003)	5′ AAUGGCGUGUGUUGGACUGUC 3′	NS3
	5′ AAGGUCACCUUUGACAGACUG 3′	NS5B
Randall et al. (2003)	5′ AACCUCAAAGAAAAACCAAACTT 3′	5′-UTR
	5′ AAGGUGCUUGUGGAUAUUUUGTT 3′	NS4B
Wilson et al. (2003)	5′ GGAGAUGAAGGCGAAGGCGUCTT 3′	NS5B
	5′ GACACUGAGACACCAAUUGACTT 3′	NS5B
Yokota et al. (2003)	5′ GGUCUCGUAGACCGUGCACTT 3′	5′-UTR
Radhakrishnan and Gartel (in preparation)	5′ GCGTCTAGCCATGGCGTTAGTATGAGTGT 3′	5′-UTR

promoter. Both siRNA-expressing vectors suppressed HCV replication, but the stem-loop type was more efficient than the tandem type.

We were able to achieve complete suppression of HCV as determined by RT-PCR in cells harboring the replicon by stably expressing short hairpin RNA (shRNA) from a VA1 fusion construct (Table 1; Radhakrishnan and Gartel, in preparation). VA1 is an adenoviral gene that has been used before for expressing ribozymes (Cagnon and Rossi, 2000) and more recently for shRNAs (Cordelier et al., 2003), both targeting the endogenous CCR5 gene as a strategy against HIV. The VA1–shRNA fusion transcripts are primarily cytoplasmic (Cagnon and Rossi, 2000), which is an important advantage because the process of RNA interference is believed to be restricted to the cytoplasm (Zeng and Cullen, 2002).

These data suggest the use of RNAi to inhibit HCV is feasible in a replicon system and selection of the targets may be easily done in this system. In addition, these data imply that target cells for HCV infection contain all the functional components that are necessary for RNAi and at this time it is not clear why HCV does not induce RNAi response during normal infection. One possibility is that HCV may inhibit Dicer-dependent cleavage of longer dsRNAs into approximately 21-nt siRNAs in vivo (Randall et al., 2003; Seo et al., 2003).

RNAi has also been shown to be effective in silencing HCV NS5B gene expression in vivo in adult mice. HCV NS5B gene was fused with a luciferase gene and expressed in mouse liver (McCaffrey et al., 2002). Naked siRNAs or siRNAs expressed from plasmids, designed against HCV NS5B gene, were delivered into the livers of mice by hydrodynamic transfection method and luciferase expression was measured. While the chemically synthesized siRNA reduced luciferase expression by 75%, the plasmid-based siRNA was able to achieve a 98% knockdown (McCaffrey et al., 2002).

RNAi against hepatitis B virus

Hepatitis B virus (HBV) belongs to the *Hepadnavirus* family that predominantly infects the liver. Though vaccines have been available for quite some time, it is estimated that every year approximately a million people die from HBV-related diseases worldwide. A number of patients with

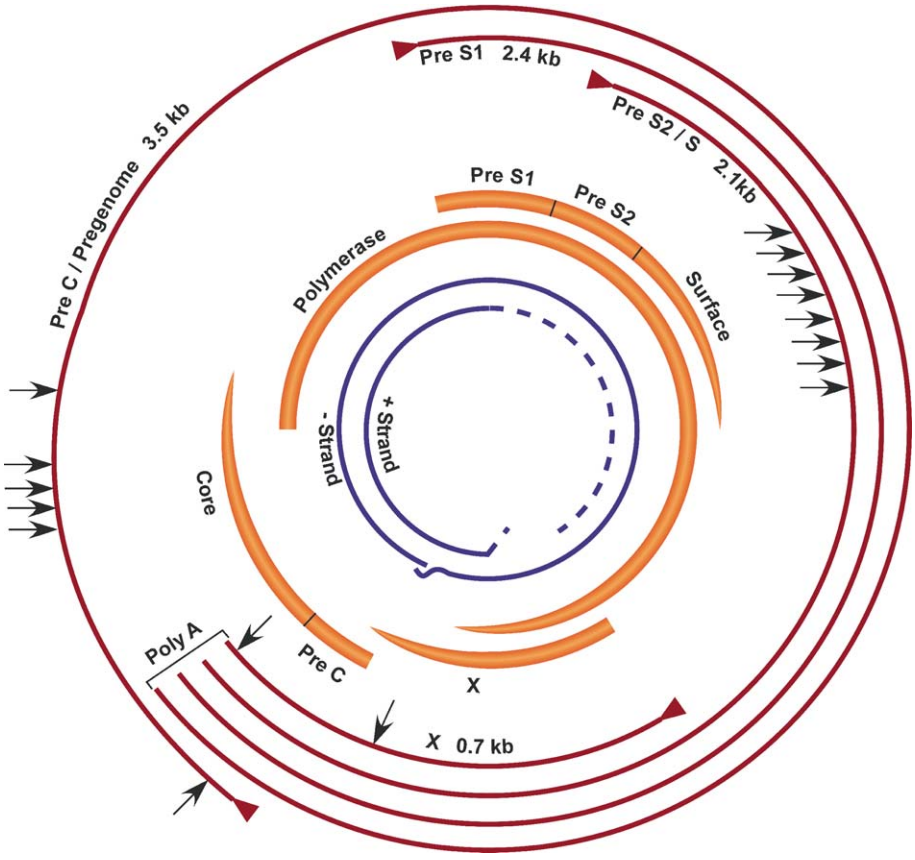

Fig. 2. Targets that have been used against HBV. The + and − DNA strands of HBV are shown in the center. The various open reading frames (ORFs) are shown around the DNA. Four outer circles represent the four different transcripts that are produced by HBV. Various targets that have been used by different groups are represented by arrows. Some of the targets are effective against multiple transcripts. For example, an siRNA targeted at the polyadenylation site will be able to knock down all transcripts because the poly A signal is common to all four transcripts.

chronic HBV infection are known to subsequently develop hepatocellular carcinoma (HCC) (Hollinger FB, 2001). The HBV genome is a 3.2-kb partially double-stranded DNA (Fig. 2) that has a core gene, which encodes for precore (pre C or HBeAg) and core (also-called C or HBcAg), the polymerase gene, which gives rise to reverse transcriptase (RT) (also-called polymerase or P), the surface protein gene, which codes for preS1, preS2, and S (also referred to as HBsAg), and the X gene, which gives rise to X protein. Four different mRNAs that code for the above-described proteins are produced from the HBV DNA that are of sizes 3.5, 2.4, 2.1, and 0.7 kb, respectively (Ganem and Varmus, 1987). The 3.5-kb transcript, in addition to coding for the preC and HBcAg, also serves as a template for reverse transcription.

At least two different treatment options have been considered for antiviral therapy of chronic hepatitis B infection. The first is the use of interferon-α that has been found to be effective only in limited cases (Lee, 1997). The other approach is to use nucleoside analogs such as lamivudine (3TC), adefovir, famciclovir, and penciclovir. Though these agents successfully reduce viral loads to undetectable levels, sustained virologic responses have been less than satisfactory (Liaw, 2002). Alternative strategies are therefore necessary to combat this disease. Again, RNA interference is an attractive option and its efficacy against HBV replication has been demonstrated recently by several groups as discussed below. The major targets used in these studies are summarized in Table 2.

Konishi et al. (2003) were able to inhibit viral replication by using chemically synthesized siRNA in a human hepatoblastoma cell line that constitutively produces HBV-infectious particles. The efficacy as measured by the secretion of HBsAg into culture media varied with the target chosen—78% with HBV-specific polyadenylation region as the target and 42% if the target was the surface region. This shows that target selection plays an important role in the success of RNAi process; but no systematic method is available yet to predict which targets would be more effective than the others. Random siRNAs used as a control were found to be ineffective demonstrating the remarkable specificity of the RNAi mechanism.

Hamasaki et al. (2003), using siRNA against the core region of HBV co-transfected with the full-length HBV DNA into Huh-7 and HepG2 cells, showed that HBeAg levels in the cell culture medium decreased about 5-fold. Also, a Southern blot for the levels of replication intermediates showed a decrease when compared to control siRNAs against GFP.

Ying et al. (2003) used two different inducible cell lines—one that produces wild-type HBV, while the other produces lamivudine-resistant HBV. They were able to show a dose-dependent reduction in replication as assessed by real-time quantitative PCR in both cell lines when siRNAs against the core region were used.

Shlomai and Shaul (2003) used a plasmid-based RNAi approach against HBV. Here, the short hairpin RNA (shRNA) was expressed under the control of H1 RNA promoter from a plasmid. The targets were chosen on the Core and X genes. Using the core and X gene expression vectors along with the RNAi constructs, they were able to show a significant reduction in the levels of these proteins as estimated by Western blots. The specificity of these siRNAs was demonstrated by using sequences with mutations that failed to suppress expression of these proteins. The same siRNA constructs were also effective against HBV replication in a cell line that constitutively expresses HBV viral particles.

Klein et al. (2003) took the next step by demonstrating that siRNAs against HBV are effective in vivo in mouse models. First, they were able to establish mice that produce HBsAg and HBeAg in the serum by injecting replication-competent HBV DNA vector via the tail vein. Also, they confirmed viral replication in hepatocytes both by RT-PCR for viral mRNA and by staining for HBsAg and HBcAg. When HBV vector was co-injected with siRNAs against either the core gene or the surface protein gene, the HBsAg

Table 2
Different targets that have been used against HBV

Authors	Target sequence	Target location
Konishi et al. (2003)	5′ ACCCTTAUAAAGAATTTGG 3′	HBV polyadenylation
	5′ GCTGTGCCTTGGGTGGCTT 3′	Pre C
	5′ TACCGCAGAGTCTAGACTC 3′	Surface
Hamasaki et al. (2003)	5′ CATTGTTCACCTCACCATA 3′	Core
Ying et al. (2003)	5′ AAGACCTAGTCAGTTATG 3′	Core
Shlomai and Shaul (2003)	5′ GATCAGGCAACTATTGTGG 3′	Core
	5′ GGTCTTACATAAGAGGACT 3′	X
Klein et al. (2003)	5′ AAGCCTTAGAGTCTCCTGAGC 3′	Core
	5′ AATTTGTTCAGTGGTTCGTAG 3′	Surface
McCaffrey et al. (2003)	5′ CTCAGTTTACTAGTGCCATTTGTTC 3′	Surface
	5′ CCTAGAAGAAGAACTCCCTCGCCTC 3′	Core
Giladi et al. (2003)	5′ CATCACATCAGGATTCCTA 3′	Surface
	5′ CCTCCAATCACTCACCAAC 3′	
	5′ CCAGTACGGGACCATGCAA 3′	
	5′ GTCTGTACAGCATCGTGAG 3′	

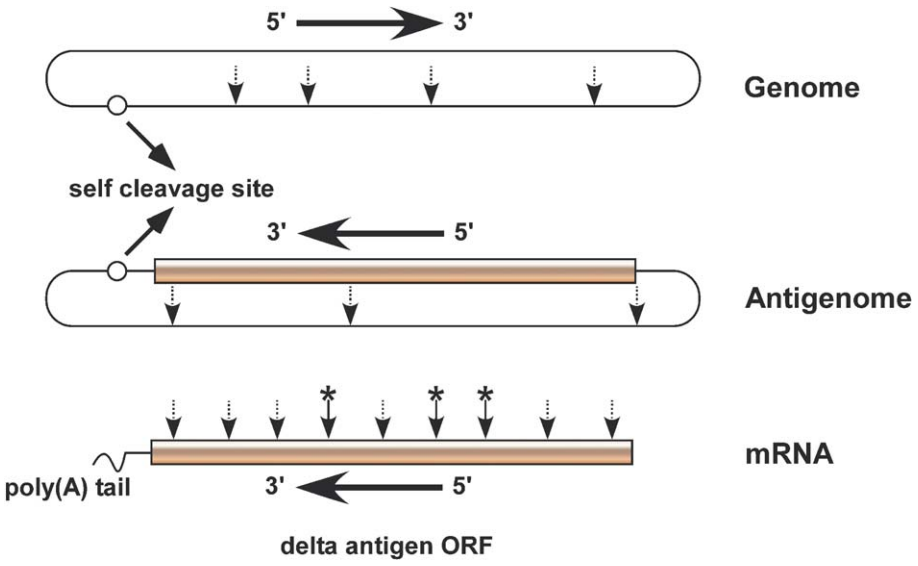

Fig. 3. Targets that have been used against HDV. The three different RNA species (genomic RNA, antigenomic RNA, and delta antigen mRNA) that are produced by HDV are shown. The targets that have been used against HDV are indicated as downward arrows. siRNAs directed against the regions shown with asterisks were the most effective (Chang and Taylor, 2003).

levels or the HBeAg levels were reduced to approximately one-third when compared to control mice injected with just the HBV vector. Also, they found that while the siRNA against the surface protein gene was able to confer long-term suppression of its target (up to 11 days), the siRNA against the core gene had a more transient response (till 3–5 days). This again clearly shows our difficulty in predicting the efficacy of siRNAs based on the sequence alone.

McCaffrey et al. (2003) used a plasmid-based RNAi approach against HBV in vivo in mouse. They used seven different targets against various regions of HBV and first showed that except one of these shRNA-producing constructs, the others were effective in reducing the amount of HBsAg levels in the culture medium of Huh-7 cells, which were co-transfected with HBV producing plasmid. Two of these shRNAs that gave more than 90% reduction after 8 days in cell culture were chosen for in vivo studies in mice. These RNAi plasmids upon hydrodynamic injection into the tail vein of the mice were able to suppress HBsAg, HBcAg, and also the viral replication. However, one of these shRNAs was shown to cause nonspecific effects in vivo. Although this shRNA was designed to target the 3.5-kb transcript of HBV, it also was able to suppress the 2.4- and 2.1-kb transcripts. Though this confers some sequence-independent antiviral activity, such nonspecific effects are least desired in RNAi-based therapy.

Giladi et al. (2003) showed that siRNAs directed against the S gene of HBV are effective against HBV both in cell culture and in mouse models in vivo as measured by HBV antigen and DNA levels. They also showed that unlike nucleoside analogues, siRNA therapy does not need active viral replication as evidenced by the reduction in HBsAg levels when mice were injected with replication-deficient HBV plasmid.

Table 3
Different targets that have been used against HDV

Target sequence	Target location
5′ AAGAAAGAAGUUAGAGGAACU 3′	mRNA for Delta antigen
5′ AAGAUAGAGGACGAAAAUCCC 3′	
5′ AACGGACCAGAUGGAGGUAGA 3′	
5′ AAGGAAGGCCCUCGAGAACAA 3′	
5′ AACAAGAAGAAGCAGCUAUCG 3′	
5′ AAGAACCUCAGCAAGGAGGAA 3′	
5′ AAGAGGAACUCAGGAGGUUGA 3′	
5′ AAGACGAGAGAAGGGAAAGAA 3′	
5′ AAACCAGGGAUUUCCAUAGGA 3′	
5′ AAAGAGCAUUGGAACGUCGGA 3′	Genomic RNA
5′ AAGGGUUGAGUAGCACUCAGA 3′	
5′ AAGCGAGGAGGAAAGCAAAGA 3′	
5′ AACUCGACUUAUCGUCCCCAU 3	
5′ AAUGCUCUUUACCGUGACAUC 3′	Antigenomic RNA
5′ AAGCGCCUCUUGUUCGCUGAA 3′	
5′ AAGUCGAGUUCCCCGGGAUAA 3′	

The sequences shown in bold were able to inhibit the HDV delta antigen by 80–95% as measured by immunoblotting (Chang and Taylor, 2003). The other sequences against the mRNA were less effective. The targets in genomic and antigenomic RNA failed to reduce HDV RNA accumulation.

RNAi against hepatitis delta virus

Hepatitis delta virus (HDV) is a 1.7-kb single-stranded circular RNA virus that replicates in the liver. Its infection in many cases has been found to lead to chronic hepatitis and also accounts for large cases of fulminant hepatitis (Hadler et al., 1992). Survival of HDV requires the presence of HBsAg supplied by simultaneous infection with HBV. In fact, the viral envelope is mainly composed of the HBsAg and a lipid bilayer. At least three different RNA species accumulate

during HDV replication—the genome, antigenome, and an mRNA that codes for the delta antigen (Fig. 3).

A study by Chang and Taylor (2003) recently showed that the delta antigen mRNA can be successfully targeted by siRNAs in cell culture (Table 3). However, the genomic and antigenomic RNAs are resistant to siRNA action. The antigenome is localized in the nucleus and so could be inaccessible for RNAi action. But the reason for the resistance of genomic RNA is unclear because a large amount of genomic RNA is cytoplasmic (Gudima et al., 2002; Macnaughton and Lai, 2002). The authors speculate that perhaps the genomic RNA could be bound by host proteins, which could protect it from RNAi action.

Conclusions and future perspectives

The studies indicated in this review show that RNAi can be used against three main hepatitis viruses—HCV, HBV, and HDV. Both cell culture experiments and in vivo studies in animals support this notion. While synthetic siRNAs were able to confer a transient response, the plasmid-based approaches have been shown to induce a more sustained response. It has been observed that siRNAs when injected via tail vein in mice have a high affinity to the liver (Lewis et al., 2002; McCaffrey et al., 2002). Because the hepatitis viruses replicate primarily in the liver of infected patients, they are attractive targets for RNAi-based therapies. Another complementary approach that has been successful against hepatitis is the targeting of cellular genes using RNAi. Song et al. (2003) showed that targeting the cell-surface receptor Fas in mouse models of autoimmune hepatitis protected it against liver damage and fibrosis.

Contrary to what was previously thought, recent evidence suggests that siRNAs and shRNAs may activate the interferon (IFN) pathway, which usually leads to nonspecific shutdown of protein synthesis and global RNA degradation (Bridge et al., 2003; Sledz et al., 2003). Using synthetic siRNAs, Bridge et al. (2003) and Sledz et al. (2003) detected no less than a 2-fold induction of 52 well-known IFN-induced genes and showed that this nonspecific effect depends on the components of the IFN pathway. Using DNA vectors that encode small RNA hairpins, Bridge et al. (2003) showed up to 500-fold induction of 2′5′-oligoadenylate synthetase (OAS1), a well established target of the IFN pathway. Their data also suggest that the ability to stimulate the IFN system depends on both the siRNA sequence and the DNA vector used. Interestingly, in one of the studies described in this review, siRNAs against HCV were specifically shown not to induce the IFN pathway (Kapadia et al., 2003). This could be explained by the choice of cell line used, as the Huh-7 and the replicon-containing cell lines used in these RNAi studies were previously shown to be defective in dsRNA signaling (Guo et al., 2003; Keskinen et al., 1999; Lanford et al., 2003). To date, no IFN-related side effects have been reported in vivo in animal studies that have used RNAi. This does not mean it is not a problem in vivo, but rather suggests that none so far have considered that possibility. Acceptable levels of interferon system activation in a therapeutic setting should be determined and the lowest effective dose of siRNA should be used because it looks plausible that the nonspecific induction of IFN by RNAi depends on the quantity of siRNA used (Bridge et al., 2003). Another recent finding is that RNAi may also have IFN-independent off-target effects in certain scenarios. Jackson et al. (2003), using a gene-expression profiling method, found that apart from the intended target, the siRNAs were able to suppress numerous other genes. Scacheri et al. (2004) made the observation that different siRNAs against the MEN1 gene were able to alter the protein levels of p21 and p53 differently, and this effect was found to be independent of the amount of siRNA used. Clearly further studies are necessary to sort out the problems of IFN-related and off-target effects before RNAi can be extended to human patients.

As with any other therapeutic strategy, efficient delivery presents a major obstacle before RNAi can be adapted to clinical trials. In principle, viral vectors could be useful in delivering shRNA-producing constructs. In the case of siRNAs, their apparent instability in the vascular system should be kept in mind. Chemical modifications of siRNAs may need to be undertaken to increase their half-life. Another approach would be to complex the siRNAs in liposomes along with a small peptide that can bind to a potential liver-specific receptor. Although the hydrodynamic tail vein injection, a method where siRNAs are infused rapidly in a volume one-tenth the mass of the animal, has been useful in the mouse system (McCaffrey et al., 2002) and has shown some initial promise in the case of nonhuman primates in delivering DNA (Zhang et al., 2001), it is far from practical in humans in a clinical setting. We will need to draw heavily from the fields of DNA-based gene therapy and antisense technology to address the issues of delivery.

RNAi is extremely sensitive to mismatches in the target regions. It has been shown that as low as a single-base mismatch can abrogate RNAi activity (Shi, 2003). It is a serious problem when RNAi is used as a therapy because viruses may become resistant by just altering a single nucleotide in the target region. This is especially true in the case of HCV where it is found that the RNA-dependent RNA polymerase (RdRP) due to its lack of the proofreading activity may introduce large number of errors during replication. This problem of escape viruses has been observed before in the case of poliovirus (Gitlin et al., 2002) and more recently for HIV (Haasnoot et al., 2003). Another possible scenario where escape viruses may arise is when the suppression of viral levels is not complete. For instance, in the case of patients with chronic hepatitis C where the circulating viral levels are between 10^5 and 10^7 genome copies/ml of blood, even if a 90% suppression of viral RNA is realized, it would still leave behind sufficient viral loads

that are prone to becoming resistant to therapy by acquiring mutations in the target region. Thus, it may be useful to target more than one region by using a combination of different siRNAs or combine different strategies such as ribozymes and antisense oligonucleotides (for a review comparing these different methods, see Scherer and Rossi, 2003) along with RNAi as a combined therapeutic approach to combat the virus.

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